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Antimicrobial Activity of Bovine Bone Scaffolds Impregnated with Silver Nanoparticles on New-Delhi-Metallo-β-Lactamase Producing *Enterobacterales* Biofilms

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Antimicrobial Activity of Bovine Bone Scaffolds Impregnated with Silver Nanoparticles on New-Delhi-Metallo-β-Lactamase Producing *Enterobacterales* Biofilms

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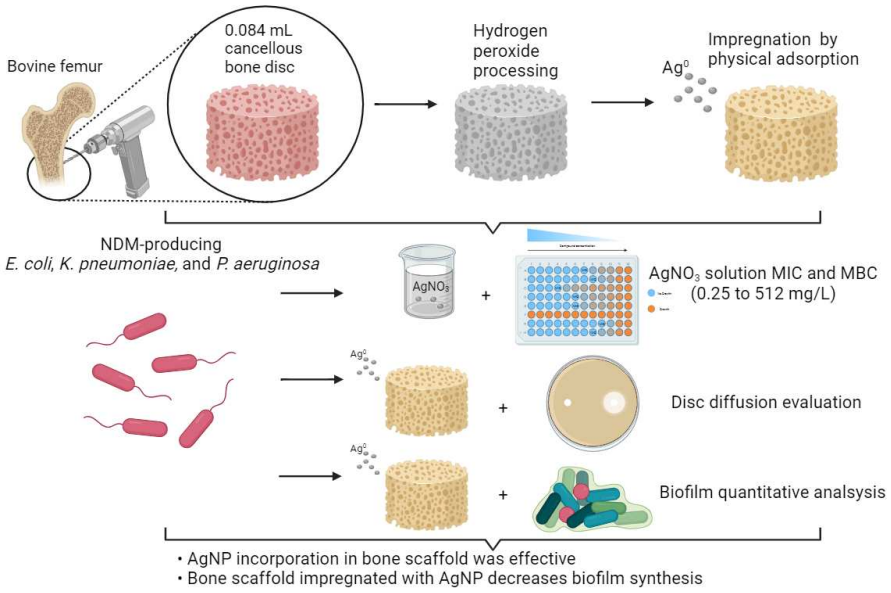
Abstract: Background: Antibiofilm activity of silver nanoparticles has been extensively investigated in common bacteria. Metallo-β-lactamase producing Gram-negative bacteria are hard-to-treat microorganisms with few therapeutic options, and silver nanoparticles were not evaluated on the biofilm of these bacteria. Objectives: The aim of this study was to evaluate the antibiofilm activity of a bone scaffold impregnated with silver nanoparticles in NDM-producing Gram-negative bacilli. Methods: Bone scaffolds from bovine femur were used for the tests and impregnated with silver nanoparticles (50 nm) by physical adsorption. Silver nitrate minimal inhibitory and bactericidal concentrations (MIC and MBC) were performed on NDM-producing *Escherichia coli*, *Klebsiella pneumoniae*, *and Pseudomonas aeruginosa*. Disc diffusion tests for silver nanoparticles susceptibility and quantification of biofilm production on plate and bone with sessile cell count were performed. Results: The MIC results demonstrated that silver nitrate had an antimicrobial effect on all microorganisms, inactivating the growth of isolates from a concentration of 8 µg/mL. MBC results showed that E. coli 16.211 was the only isolate to present MIC different from MBC, with a value of 16 µg/mL. Conclusion: Bone scaffolds impregnated with silver nanoparticles can significantly reduce biofilm, and it can be a strategic material to be used as an implant for different approaches.

Keywords: Biofilm; silver; bone; nanotechnology; impregnation; bacteria; NDM; scaffold



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1. Introduction

The emergence of NDM-1 carbapenemase (New Delhi Metallo-β-lactamase) is a global public health problem. The high hydrolysis capacity of this β-lactamase, including against carbapenems, and their association with other plasmid-borne resistance mechanisms decreases the available therapeutic options [1]. In some cases, only polymyxin and cefiderocol are active drugs against these pathogens [2, 3].

Silver nanoparticles (AgNPs) have already been studied in NDM-producing *Pseudomonas aeruginosa* isolates, however, only the AgNPs activity in solution was evaluated [4]. Furthermore, antibiofilm AgNPs activity was not still evaluated in NDM-producing microorganisms. The use of bone grafts is important in the reconstructive medicine of trauma and chronic bone diseases. Heterologous grafting is an option that has been widely used in the most varied areas of medicine. Its main representative is the bovine graft, which is easy to obtain, has great availability and is similar to human bone [5,6]. However, it may present limitations related to antigenicity [7]. With the aim of reducing antigenicity and preserving the inorganic matrix, the bovine graft undergoes washing processes, decellularization, is degreased and subsequently dehydrated. Bovine bone has chemical (composition), physical (porosity and size) characteristics and biological behavior very similar to human bone, which favors osteoconduction. In addition, its inorganic matrix provides calcium and phosphorus content, which are essential for the formation of new bone tissue at the implant site [5,8].

However, the reconstruction of bone defects is still a considerable clinical challenge since several factors can hinder the success of the procedure. It can be associated with different complications, including infection of the graft with reabsorption of the bone tissue. These infections can be caused by local microbiota or even hospital-acquired pathogens, and biofilm is the cornerstone of infection maintenance [9]. The infections, especially those caused by multidrug-resistant bacteria and bacterial biofilms are described with great relevance, as they are considered the most devastating complications of surgeries related to grafts, leading to long hospital stays, complex and prolonged treatments with local and systemic antimicrobials, new surgery at the grafting site for debridement with possible loss of the graft and adjacent tissues [10,11,12].

Several antibiotics and other substances (*e.g.* bioglass and metals) have been used to impregnate the implant surface or even the bone graft. The antimicrobial activity of AgNPs has been extensively investigated, including incorporating this metal into dental and orthopedic materials [13]. The size of the AgNPs increases the contact area with the bacterial cell membrane and causes permeability and easy penetration. Silver ions can interfere with the mitochondrial respiratory chair causing oxidative reduction. Another supposed mechanism is the osmotic imbalance caused by the difference between extracellular and intracellular environments [14]. The impregnation of bone scaffolds can decrease trans and postoperative infection rates with a high risk of failure, loss of bone volume, and chronic infectious processes, such as osteomyelitis.

There is a recent report of an experimental study concluding the non-reaction of a foreign body or inflammatory process in a bone graft impregnated with AgNPs [15]. However, the impregnation of bone graft with AgNPs has been poorly investigated, and microbiological studies are lacking in multidrug-resistant bacteria. The purpose of this investigation was to evaluate the *in vitro* antibiofilm activity of a bone scaffold impregnated with AgNPs in NDM-producing bacteria, since it present known resistance to commonly used antibiotics.

2. Materials and Methods

Study delimitation

This was an experimental study conducted with bovine bone scaffolds models impregnated with AgNPs.

Bone scaffolds processing, nanoparticles synthesis, and AgNPs impregnation

Cancellous bone discs from the bovine femur were used for the tests. The discs with 6 mm in diameter and 3 mm thick (0.084 mL) (retrieved from a sagittal cut in the bone using a table band saw, using 6 mm diamond drill, small bone cylinders) (Figure 1) were made. Then, using a ½ inch chisel, a hammer and a plastic caliper, the discs were measured and cut) were processed using a modified protocol previously described [16]. The procedure used to AgNPs synthesis was adapted from Dong

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et al. [17]. After one hour of stirring, the yellowish-colored solution containing the silver nanoparticles, 50 nm in size, was confirmed with ultraviolet light spectra and scanning electronic microscopy (SEM) as previously described [18].

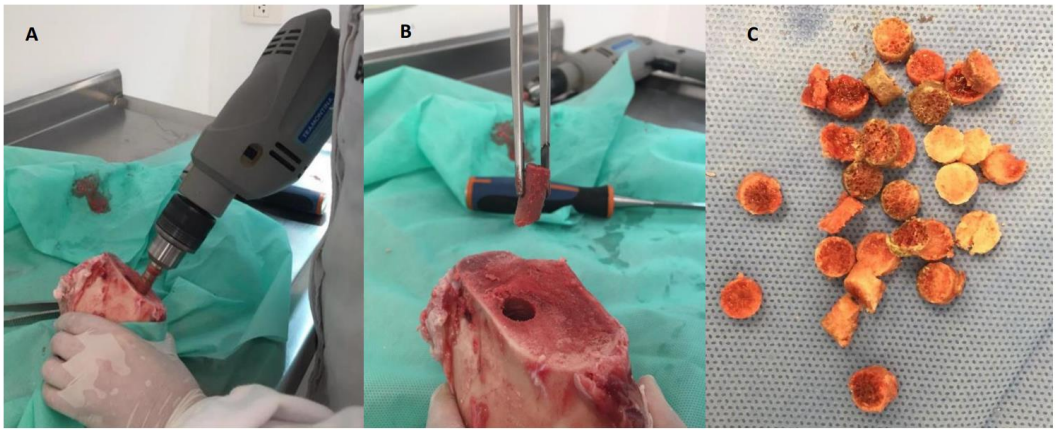

Figure 1. Bone scaffold processing. A: 6 mm diamond drill punching the sagittal cut. B: Bone cylinder after punching. C: Bone discs with 6 mm in diameter and 3 mm thick.

Physical adsorption was used to incorporate the AgNPs in the bone matrix, based on an adaptation of the model reported by Becerril-Juárez et al. (2012) [19]. For this purpose, bone models were added to the AgNPs solution at room temperature, for 60 minutes, protected from light. After the incorporation of AgNPs in the bone matrices, the samples were kept at a temperature of 70°C for 60 minutes, in an oven with no air circulation system for drying, followed by lyophilization.

Qualitative bone discs scaffolds analysis through Scanning Electronic Microscopy and Energy Dispersion X-Ray Spectroscopy

For the analysis of morphology and microstructural characteristics before and after AgNPs impregnation in the bone matrix, SEM was performed. The models were transferred to sterile glass Petri dishes with the primary fixative agent (0.68 g 99.5% sucrose, 0.42 g 98% sodium cacodylate, 0.6 mL 30% glutaraldehyde (Merck, Darmstadt, Germany) in 19.4 mL of deionized water, remaining in contact for 45 minutes. After contact with this primary fixative agent, the models were transferred every 10 minutes to the following solutions: buffer (composed of sucrose and cacodylate of sodium at the concentrations mentioned above), 35% ethanol, 50% ethanol, 70% ethanol, 100% ethanol and HMDS PA (hexamethyldisilazane) (Merck, Darmstadt, Germany). After fixation, the models were kept in a desiccator until the moment of visualization in SEM, when they were previously submitted to metallization with gold particles, in metallization equipment with a Q150R ES rotary pump (Quorum Technologies, Lewes, United Kingdom) and, later, fixed on a metallic base for observation in the scanning electron microscope JEOL JSM 6010PLUS-LA at an accelerating voltage of 20 kV. Observations were made at magnifications between 2,000 and 100,000 times [18].

Energy Dispersion X-Ray Spectroscopy (EDS) allows the measurement of the chemical elements present in the sample as well as determination of concentrations with great precision. The bone discs were characterized by EDS, before and after AgNPs impregnation in the bone matrix, in order to verify the presence and dispersion of silver on the bone tissue. EDS analysis was performed on the JEOL JSM 6010PLUS-LA Analysis Station with an accelerating voltage of 20 kV.

Microorganisms, Silver nitrate Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC), Disc diffusion tests for AgNPs susceptibility, and Biofilm production and evaluation on bone

Three NDM-producing bacteria were included in this analysis, one *Klebsiella pneumoniae* 11.955, one *Escherichia coli* 16.211, and one *P. aeruginosa* 20.589. The identification was confirmed by MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry) (Bruker Daltonik, Bremen, Germany), and *bla*NDM gene was identified and confirmed by whole gene sequencing (MiSeq®, Illumina, San Diego, CA).

There is not a validation of MIC (Minimal Inhibitory Concentration) for AgNP. Thus, silver nitrate MIC was performed as previously described [20], following the principles stablished by CLSI [21]. A silver nitrate solution in progressive titers from 0.25 to 128 mg/L was used. 200 µL aliquots of silver nitrate solution in Muller-Hinton broth in different concentrations were placed in a 96-well plate and 5 µL of the solution of each microorganism were inoculated to reach a concentration of 106 colony forming units (CFU)/mL. After incubation for 24 hours at 36°C, the MIC was defined as the lowest concentration that did not show bacterial growth. All tests were performed in triplicate. In parallel, the minimal bactericidal concentration (MBC) for AgNPs was performed with the same microorganisms above, plating in triplicate 100 uL of all solutions over the MIC. The MBC was determined as the final concentration without bacterial growth.

The microorganisms were previously diluted to a turbidity standard equivalent to 0.5 McFarland and inoculated on Mueller-Hinton agar plates. In addition, two discs, one without impregnation (negative control) and one with AgNPs (test), were transferred to Muller-Hinton agar plates inoculated with different bacteria. The plates were incubated for 24h at 36 °C. The analysis was quantitative, checking for the presence and measuring the diameter of inhibition halo [22, 23].

For biofilm production, we used protocols previously described [24, 25]. The experiment was executed in quintuplicate. From each microbe suspension, a 1:10 dilution was made in TSB until concentrations of 10^7 CFU/mL of bacteria were achieved. Then, 10 mL of TSB was poured into sterile 12-well plates until it covered completely the bone discs (control – without AgNPs after processing and AgNPs impregnated) for 2 hours under agitation (120 rpm), so that cells could adequately adhere. The specimens were transferred to a new sterile 12-well plate containing 0.9% NaCl to remove planktonic cells from the material. Then, specimens were transferred to another sterile 12-well plate and submerged in 10 mL of TSB at 37°C for 24 hours without agitation. During this step, the cells adhered to the device surface formed the biofilm. After this step, the specimens were submerged into 50 mL conical tubes filled with 10 mL of sterile 0.9% NaCl to remove the residues and unadhered/planktonic cells (step I). After this washing step, the specimens were allocated into 50 mL conical tubes filled with 10 mL of 0.9% NaCl for further processing (sonication), and the liquid of the last washing was stored for planktonic cells analysis (step II).

Five specimens of each group were transferred to sterile conical tubes with 10 mL of 0.9% NaCl and sonicated for 15 minutes in an ultrasonic bath using a Soniclean 15 (Sanders Medical, Santa Rita do Sapucaí, Brazil) at a frequency of approximately 40 kHz and temperature of 35°C [26]. After the sonication step (step II), the supernatant (100 µL) was inoculated in TSA for growth evaluation and cells count (CFU/mL).

SEM and EDS data were descriptive and semi-quantitative, respectively. MIC and MBC were presented in µg/mL of silver nitrate. AgNPs activity data by agar diffusion were presented in millimeters (mm) and defined as presence or absence of inhibition halo. For comparing the cell count in the groups, the median CFU/mL obtained with quantitative culture was analyzed by the Mann-Whitney test and presented with a median with an interquartile range (25-75%). The difference in CFU/mL was significant when $p < 0.05$. The data was calculated, analyzed, and plotted using Prism 7.0 (Graphpad, San Diego, CA).

3. Results

3.1. AgNPs impregnation, Qualitative bone discs scaffolds analysis through Scanning Electronic Microscopy and Energy Dispersion X-Ray Spectroscopy

The procedure used to obtain AgNPs produced a yellowish color observed with a maximum absorption peak around 420 nm, measured by ultraviolet light spectra (Figure 2), suggesting the formation of AgNPs of about 50 nm in diameter. Using SEM and EDS, it is possible to observe the presence of nanoparticles impregnated in the bone scaffolds (Figure 3) due to the presence of two characteristic peaks of silver atoms, corresponding to 18.59% (w/w) of the sample. From EDS, one can confirm that the physical adsorption procedure used here was efficient in impregnating AgNPs and keeping them attached after drying the scaffolds (Figure 4).

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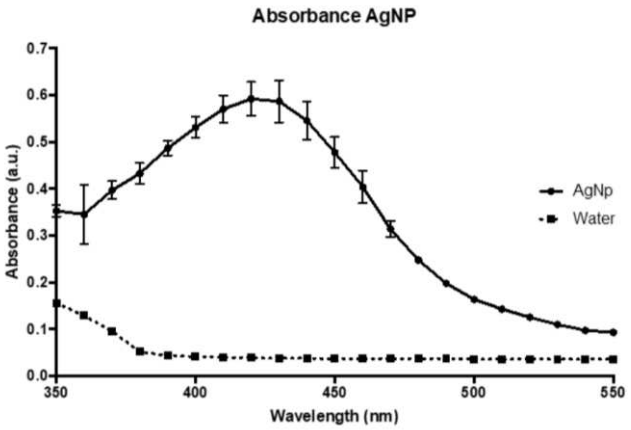

Figure 2. Absorbance curve of the AgNPs solution, showing a peak at 420 nm.

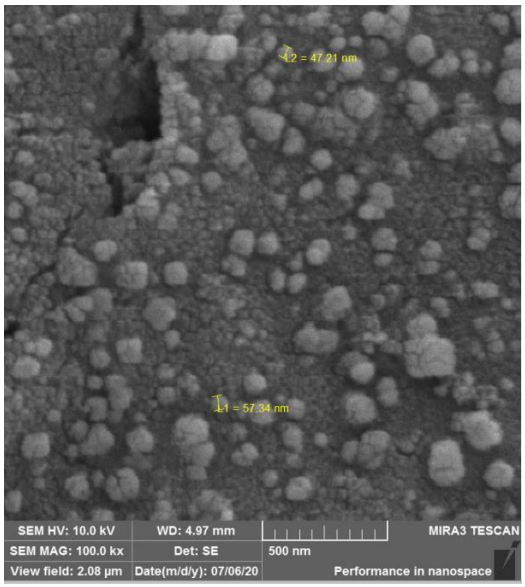

Figure 3. Scanning Electron Microscopy showing the morphological analysis of AgNPs on the bone surface, at 100,000 times magnification. It was possible to visualize the AgNPs impregnated after treatment.

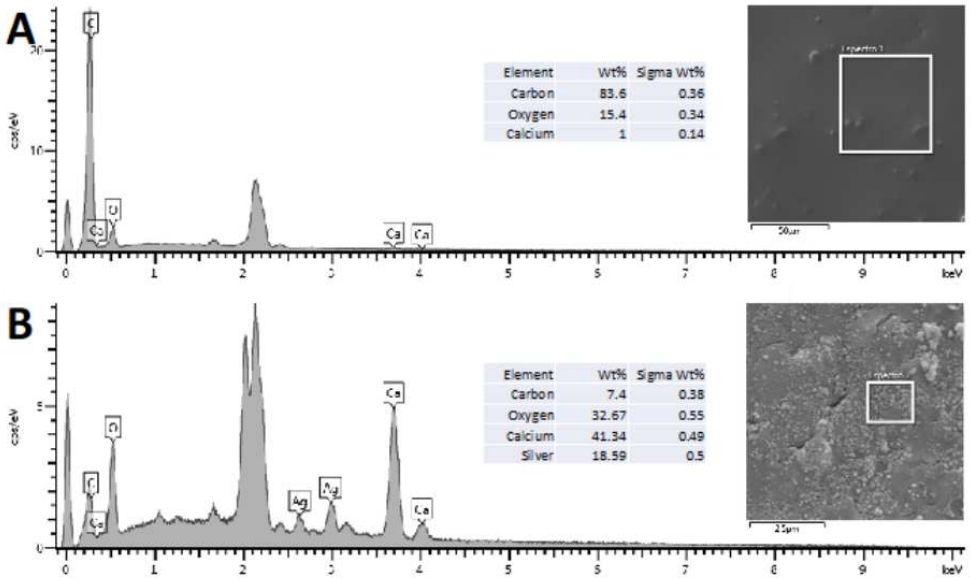

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Figure 4. Graph resulting from the EDS analysis quantifying chemical components (C = carbon; O = oxygen; Ca = calcium; Ag = silver) of the model impregnated with AgNPs. A: Control, before AgNPs impregnation in the bone matrix. B: Test, after AgNPs impregnation in the bone matrix.

3.2 Silver nitrate Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC), Disc diffusion tests for AgNPs susceptibility, and Biofilm production and evaluation on bone

The MIC results demonstrated that silver nitrate had an antimicrobial effect on all microorganisms, inactivating the growth of isolates from a concentration of 8 µg/mL. MBC results showed that *E. coli* 16.211 was the only isolate to present MIC different from MBC, with a value of 16 µg/mL.

It was possible to identify the presence of an inhibitory halo in the agar plates of all tested microorganisms. *E. coli* 16.211 halo size was 15 mm, and in *P. aeruginosa* 20.589 it was 17 mm. The *K. pneumoniae* 11.955 plaque showed two inhibition halos, the internal one measuring 11 mm and the external one measuring 13 mm.

AgNP-impregnated bone scaffolds showed a significant reduction in biofilm cell development (> 3log CFU/mL) for *E. coli* (p = 0.0078) and *P. aeruginosa* (p = 0.0079). However, there was no significant decrease for *K. pneumoniae* (p = 0.5476) (Figure 5).

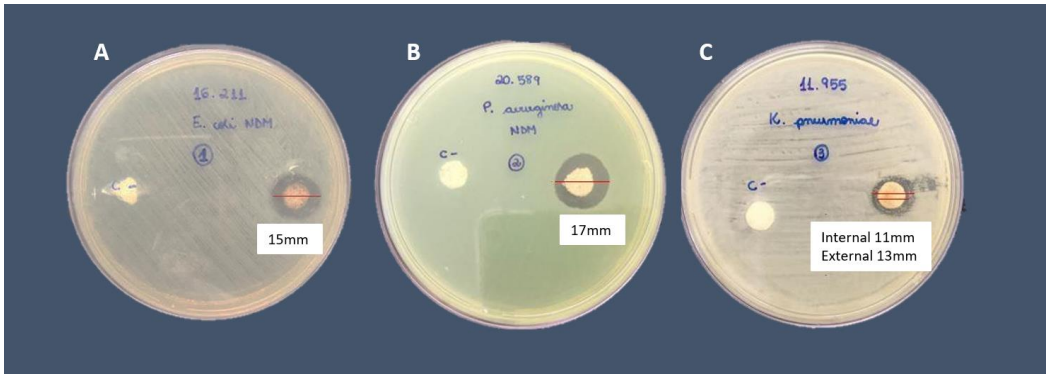

Figure 5. Antimicrobial activity of AgNP-impregnated bone scaffolds disc against A: E. coli 16.211, B: P. aeruginosa 20.589, and C: K. pneumoniae 11.955.

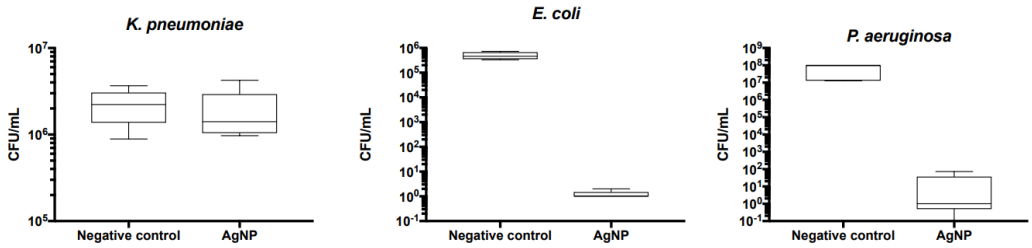

Figure 6. Cell count (CFU/mL) of biofilm of each evaluated microorganism in bone grafts with AgNPs and without impregnation (control). .

4. Discussion

This study identified that three NDM-producing bacteria were susceptible to silver, but biofilm inhibition presented different results. Even so, it is important to identify that the bone scaffold impregnated with AgNPs may be an attractive graft against this type of microorganism, where therapeutic options may be null, and radical orthopedic procedures may be necessary, but with significant sequelae for patients, like limb amputation [27].

Even though all isolates were susceptible to silver, these values were obtained by silver nitrate, differently from other authors who defined MIC by AgNPs, showing divergent results [28]. Mathur et al. described the antibacterial action of AgNPs through penetration into the microorganism's cell membrane, increasing its permeability, leading to the rupture of this structure and consequent cell death [29]. Another mechanism described is the formation of free radicals that damage the cell membrane, causing porosity and cell lysis [30]. Mih et al. analyzed the mechanism of resistance to

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oxidative stress of *E. coli* K-12 MG1655 strain, punctuating hypothetically the presence of amino acids capable of absorbing oxidative damage in the structural proteome of this bacterium [31].

Along with the bactericidal mechanism of silver nanoparticles, the relationship between particle size and antimicrobial effect has been reported [32]. Smaller particles have a higher surface area that allows the release of a higher amount of silver ions, responsible for the most acceptable mechanism of bactericidal action. In this sense, smaller particles are expected to present a greater antimicrobial effect than bigger particles and particle aggregates. This can be explication for the maintenance of viable bacterial cells after scaffold impregnation, in the case of *K. pneumoniae*. Bacteria with a high capacity of multiplication and biofilm-producing, like *P. aeruginosa*, showed higher susceptibility to AgNPs.

Biofilm formation is a variable between microorganism species [33]. Olson et al. highlighted that *P. aeruginosa*, *E. coli*, and *Staphylococcus aureus* produce biofilms in a short period and without great nutritional requirements [34]. However, in our study, the broth used in the biofilm model presents abundant nutrients, which can differ the biofilm pattern and bacterial redox system, which can affect silver activity [35]. Anuj et al. evaluated the association of silver nanoparticles with linezolid as a bactericidal agent on *E. coli* MTCC® 443 ™, where the resistance mechanism of drug efflux of this microorganism was inactivated by AgNPs [36]. This is an important issue, considering that AgNPs can be an important molecule to be used in combination with antibiotics.

5. Conclusions

This study has some limitations because we evaluated a few bacteria and *in vitro* model cannot represent a real-life biofilm in osteomyelitis. Even though the literature is broad on impregnated materials, microbiological studies with biofilm on bovine scaffolds were not reported yet.

Considering the current results of our study, this bone scaffold has both antimicrobial and anti-biofilm properties for two NDM-producing microorganisms. AgNPs incorporation was effective, which must be confirmed in in vivo models. Bone scaffold impregnated with AgNPs decreases biofilm synthesis by reducing the viability of sessile cells, being a promising material in the clinical use of dental and orthopedic procedures. In vivo studies are needed to assess osseointegration, anti-biofilm efficacy, and safety.

Author Contributions: G.A.G. – Conceptualization, microbiological studies, writing of the draft; V.S.T.R. – Microbiological studies, manuscript writing and review; L.R.D. – Microbiological studies, results analysis; A.P.A. – Microbiological studies, results analysis; P.H.S. - Microbiological studies, results analysis; M.A.W. - Nanoparticles' processing and impregnation; F.F.T. – Manuscript writing and review, data analysis.

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Conflicts of Interest: F. F. T. is a CNPq researcher. The other authors declare no conflict of interest.

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